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1. Background

X-linked lymphoproliferative disease (XLP) is an inheritable disorder of the immunological system. The main clinical feature of XLP is represented by a fulminant infectious mononucleosis triggered by Epstein Barr virus (EBV) and followed by lymphoproliferation, organ failure and death, and in some cases lymphomas and autoimmunity[1]. About 60% of the cases (XLP-1) are due to mutations of SLAM-associated protein (SAP) gene SH2D1A on chromosome Xq24-25, encoding an adaptor protein containing a single SH2 domain with essential functions in the regulation of immune system. SAP mutations found in XLP-1 are diverse, all resulting in the absence or instability of the protein or in the impairment of its adaptor function[2].

SAP is mostly expressed in T cells, NK cells, iNKT cells and some B cells. Through its Src homology 2 (SH2) domain SAP binds specifically to tyrosine-based motifs T/SIpYXXV/I in the cytoplasmic region of SLAM-family receptors [3,4]. Its best characterized function is the recruitment to the active SLAM receptor of the tyrosine kinase FynT; this recruitment is necessary for the complete receptor phosphorylation and it controls downstream signaling[5,6]. However, based on the single SH2-domain organization of SAP-related adaptors, many reports postulate that SAP might carry out an inhibitory function by binding phosphorylated SLAM-family tyrosine residues and competing with effectors such as lipid (SHIP1) and tyrosine phosphatases (SHP-1 and SHP-2)[7].

A number of recent reports demonstrate that, besides SLAM family receptors, SAP interacts with other signaling proteins such as the CD3ς chain[8], the adhesion receptor PECAM-1[9], the Lyn tyrosine kinase[10], the Nck1 adaptor[11] and βPIX, a guanine nucleotide exchange factor for Rac and Cdc42[12].

SAP absence compromises several compartments of the immune systems, impairing the T cell helper function, the $CD8^+$ and NK cytotoxicity toward EBV infected targets and the development of iNKT population[1]. T cell of XLP patients were reported to feature defects in antigen-stimulated proliferation, IL-2 secretion and homotypic cell aggregation, due to an imbalance of early TCR signaling[8]. Moreover, SAP deficiency has been shown to impair the ability of antigen-stimulated $CD4^+$ T cells to organize the immune synapse and form stable interactions with cognate B cells in the germinal center[13,14]. The reduction of such interaction couples with a defect of the SAP-Fyn-PKCθ-Bcl10-NFkB pathway, resulting in deregulated cytokines production and incapacity to deliver helper signals to B cells leading to hypo-dys-gammaglobulinemia[15-18].

Recently a strong connection between SAP and apoptosis has emerged. Indeed two reports describe XLP-like diseases in patients due to mutations in Itk, a tyrosine kinase regulating DG-signaling and cell survival[19] and in XIAP (X-linked Inhibitor of Apoptosis)[20], suggesting that an important component of XLP is the inability of the cell to undergo controlled cell death [21]. Accordingly SAP is a direct target of p53 and mediates p53-induced apoptosis of lymphoblastoid cell lines, justifying the increased occurrence of tumors in XLP patients [22-24]. Finally strong defects in restimulation induced apoptosis (RICD) have been documented in T cells from XLP patients[25]. RICD is a programmed cell death form that occurs as a result of repeated stimulation of TCR and helps to maintain the peripheral immune tolerance[26]. In XLP patients, the defective RICD couples with a defective clearance of infected cells and a persistent viral infection, thus sustaining acute lymphocyte hyper-proliferation triggered by recurrent antigen encounters[22,25]. The defective RICD is due to the decrease of TCR signaling in SAP deficient cells, resulting in resistance to the induction of key pro-apoptotic genes such as FASLG (Fas ligand) and BCL2L11 (Bim)[25]. Similarly, lymphocytes from SAP KO mice feature reduced sensitivity to activation induced cell death (AICD) due to reduced p73 induction and impaired caspase activation[27].
Diacylglycerol kinases (DGKs) are a family of 10 enzymes that phosphorylate diacylglycerol (DG) to phosphatidic acid (PA), with a highly conserved catalytic domain preceded by two cysteine rich C1 domains and different regulatory domains[28]. By regulating in a reciprocal and highly compartmentalized manner the levels of those lipid second messengers, DGKs act as terminators of DG-mediated signals and activators of PA-mediated signals. The pivotal role of DGKs in the termination of DG signals is showed by the KO of several DGKs isoforms resulting in the enhancement of DG-mediated signaling and over-activation of either DG-regulated PKC and Ras-GRP proteins, the latter leading to either Ras or Rap activation[29-31]. In particular in T cells two isoforms (DGKα and DGKς) have a master role in regulating cell sensitivity to TCR activation by negatively modulating the intensity and the kinetic of DG-mediated signaling[32]. However the two isoforms are subjected to differential regulation, with DGKς playing a major role in DG metabolism at the immune synapse while DGKα seemingly poorly active despite high expression levels[28]. Accordingly with a key role of DGKα in controlling TCR responses, its expression is down-regulated within few hours from T cell activation[33] while its overexpression contributes to anergy onset [34,35]. DGKs activity is regulated also at a post transcriptional level with evidences of both activation by growth factors[36] and cytokines[37] but also inhibition by GPCR[38] or cell stress[39].
Investigating DGKα regulation in the early phase of T cell activation, we showed that strong TCR agonist induces a rapid negative regulation of DGKα enzymatic activity and a slower accumulation at the membrane, whereas DGKς remains active and translocates to the immune synapse. The inhibition of DGKα is still poorly characterized at a molecular level but requires PLC activity, Ca^{++} and SAP, the adaptor protein mutated in XLP-1 disease. Accordingly, SLAM triggering or overexpression of SAP are sufficient to inhibit DGKα, whereas SAP mutants unable to bind either phosphotyrosine residues or SH3 domains are ineffective[40].
When Jurkat cells are made SAP deficient, DGKα is maintained active and metabolizes DG impairing MAPK pathway and NFAT activity, decreasing IL-2 production. Those data suggest that DGKα activity needs to be inhibited by SAP to allow the accumulation of DG required for efficient signaling and full T cell activation[40]. The idea that DGK activity needs to be inhibited to allow robust DG accumulation and signaling is consistent with recent studies by the principal investigator and others on additional DGK isoforms[41].

2. Project aim/objectives

In here we work on the hypothesis that the lack of SAP in T cells of XLP patients should result in an improperly active DGKα and an increased DG metabolism, contributing to the reduced TCR signal

intensity and to the RICD resistance. Our data indicates that the potentiation of TCR-induced DG signaling by DGKα knockdown or DGK inhibition allows the TCR signaling to reach the threshold required for RICD onset *in vitro*. Those findings elucidate the relevance of the functional interaction between SAP and DGKα in the control of lymphocyte homeostasis and support the possible application of DGKα inhibitors for XLP treatment.

3. Experimental plan and methods

Cells culture and reagents

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood or buffy coat from normal subject or XLP patient by Ficoll-Paque PLUS (GE Health Care) density gradient centrifugation, washed, and resuspended at $2*10^6$ cell/ml in RPMI-GlutaMAX (GIBCO, Life technologies) containing 10% FCS heat inactivated, 2 mM glutamine, and 100 U/ml each of penicillin and streptomycin (Invitrogen). T cells were activated with 1 µg/ml of anti-CD3 (clone UCHT1) and anti-CD28 (clone CD28.2) for 3 days. After 3 days, activated T cells were thoroughly washed and then cultured in complete RPMI-GlutaMAX supplemented with 100 IU/ml rhIL-2 (Peprotech) at $1.2*10^6$ cells/ml for at least 7 days before apoptosis assays were conducted (the media was changed every 2-3 days).
R59949 and R59022 (Sigma-Aldrich) was dissolved in DMSO; equal amounts of DMSO were used in the control samples. All reagents are from Sigma-Aldrich apart: human recombinant IL-2 (Peprotech), 1,2-dioctanoyl-sn-glycerol (DG 08:0, # 800800O, Avanti)

Antibodies

Antibody	Vendor	Type
anti-actin	Santa Cruz	monoclonal
anti-tubulin	Sigma-Aldrich	monoclonal
anti-DGKα	Abcam	polyclonal
anti-human CD3ε (OKT3)	in house	monoclonal
anti-human CD3 (UCHT1)	eBiosciences	monoclonal
anti-human CD28 (CD28.2)	eBiosciences	monoclonal
anti-SAP	Cell Signaling	polyclonal
anti-TAC IL-2Rα	Abcam	monoclonal
Anti-mouse Alexaflour 488	Life Technologies	polyclonal
anti-mouse HRP	Perkin Elmer	polyclonal
anti-rabbit HRP	Perkin Elmer	polyclonal

siRNA for transient silencing

Activated human PBLs were prepared as described above and transfected with 200 pmol of siRNA oligonucleotides specific for the target protein (Stealth Select siRNA; Life Technologies) or a NS control oligo (Life Technologies). Transient silencing was obtained by transfection using the Amaxa nucleofector kit "Human T cell nucleofector kit" (VPA-1002 Lonza) and the Amaxa Nucleofector II system (program T-20, Lonza) according to manufacturer instructions. The cells were maintained in culture in presence of IL-2 (100 IU/ml) for 4 day to allow target gene knockdown.

siRNA	Sense strand	Antisense strand
SAP	UGUACUGCCUAUGUGUGCUGUAUCA	UGAUACAGCAGACAUAGGCAGUACA
DGKα	CGAGGAUGGCGAGAUGGCUAAAUAU	AUAUUUAGCCAUCUCGCCAUCCUCG
PKCθ	CGUUGGAUGAGGUGGAUAAtt	UUAUCCACCUCAUCCAACGga
RasGRP1	CUACGACAAUUACCGGCGAtt	UCGCCGGUAAUUGUCGUAGtt

Stealth RNAi Negative Control Duplexes (# 12935-300, Life Technologies) was used as negative control.

Cytofluorimetry

To test the restimulation induced cell death (RICD), activated T cells (10^5 cells/well) were plated in triplicate in 96-well round-bottom plate and treated with anti-CD3ε mAb OKT3 (1-2-5-10-50-100ng/ml) in RPMI-GlutaMAX supplemented with 100 IU/ml rhIL-2 for 24 hours. In some assays R59949/ R599022 (5-10 μM) or DG (50 μM) were added 30 minutes before the restimulation. 24 hours after treatment, cells were stained with 1 μg/ml propidium iodide and collected for a constant time of 30 seconds per sample on a FACScan flow cytometer (FACS calibur, BD). Cell death was analyzed with CellQuest software (BD) or Flowing software (Turku Bioimaging) as percentage of cell loss = (1 – [number of viable cells (treated) / number of viable cells (untreated)]) × 100.

Alternatively, for the IL2R assays, 10^6 cells/experimental condition were stimulated (100ng/ml) for 24 hours and then the cells were fixed by using the FIX & PERM CELL PERMEABILIZATION REAGENT kit (Invitrogen). After the samples were incubated with anti-TAC IL-2R α (0.5 μg) 20 minutes in the dark and subsequently with the secondary anti-mouse Alexaflour 488. 10^5 cells were collected on a FACScan flow cytometer (FACS calibur, BD).

Quantitative RT-PCR

The activated lymphocytes were stimulated at density of $30*10^6$ cell/ml in RPMI-GlutaMAX 10% FCS H.I. with 10 μg/ml anti-CD3ε clone OKT3 for 4 hours. R59949 (5 μM) was added 30 minutes before the restimulation. The restimulation was stopped by washing the cells with cold (4°C) PBS and the mRNA was extracted by ChargeSwitch Total RNA Cell Kit (Life Technologies). The RNA concentration and purity were estimated by a spectrophotometric method using NanoDrop 2000c (Thermo Scientific). The RNA was retrotrascribed with High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) and cDNA quantified by real time PCR (C1000 Thermal Cycler CFX96 realtime system, BIORAD) using GUSB as normalizer.

TaqMan gene expression assays were from Life Technologies: Hs00939627_m1 (glucuronidasi beta, GUSB), Hs00158978_m1 (SH2D1A), Hs00176278_m1 (DGKα), Hs00174114_m1 (IL-2), Hs00989291_m1 (INFγ), Hs 00374226_m1 (NR4A1) and Hs 00545007_m1(NR4A3).

Statistics

Data are shown as the mean ± SEM. For statistical analysis, Student's t-test or *ANOVA* were used. Experiments shown are representative at least 3 independent experiments. A *p*-value less than or equal to 0.05 was deemed to be significant in all experiments.

4. Results

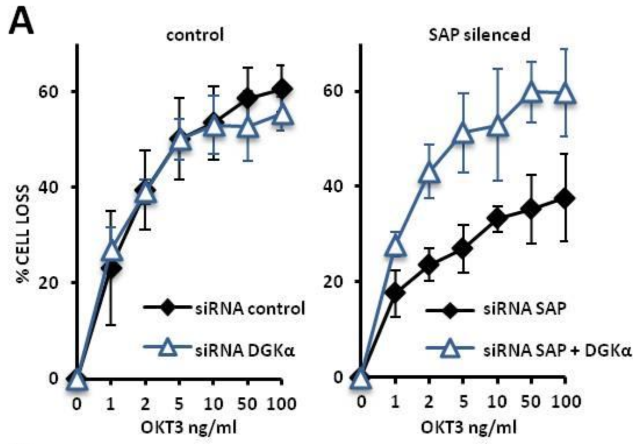
1) DGKα silencing or inhibition rescues RICD in T cell both from XLP patient and in SAP deficient cells

A reduced TCR signaling strength promotes the resistance to RICD of XLP patients' SAP deficient lymphocytes[25] thus we investigate if DGKα knockdown could rescue cell death in SAP deficient cells.

To verify such hypothesis we first used PBLs from healthy donors in which SAP function was downregulated by RNAi-mediated silencing. SAP silencing results in a severely reduced RICD over a wide concentration-range of anti-TCR agonist antibody ([25] and Fig. 1A and 1B). While DGKα silencing does not affect RICD in control cells, it fully restores agonist induced cell death in SAP silenced cells (Fig. 1A) demonstrating an essential and selective role of DGKα in the resistance to cell death of SAP deficient cells. To verify the relevance of excessive DGK enzymatic activity in the onset of RICD resistance in SAP deficient cells, we used two well characterized inhibitors featuring a discrete selectivity for the α isoform: R59949 and R59022[52]. Both of them marginally affect RICD in control cells, while fully restoring TCR-induced death in SAP deficient cells at 5 and 10 μM (Fig. 1B and 1F), while lower concentrations tested are ineffective (data not shown). Similarly, DGK inhibitor R59949 restores TCR induced apoptosis in lymphocytes from SAP KO mice without affecting cell viability in lymphocytes from WT mice (data not shown).

To confirm the relevance of those findings in XLP-1 patients we compared PBLs' patient to those derived from healthy donors in this assay. As previously shown DGKα silencing or inhibition did not affect the robust RICD in lymphocytes from healthy donors. Conversely, XLP patients feature various degrees of RICD impairment[25], which is fully restored upon DGKα silencing (Fig. 1C). To verify the contribution of DGK activity to RICD resistance in XLP patient T cells we tested the DGK inhibitors R59949 and R59022, observing a partial restoration of RICD at concentrations of 5 and 10 μM (Fig. 1D and 1E). These data suggest that RICD resistance in XLP-1 is due to constitutive DGKα activity and that targeting DGKα restores RICD *in vitro.*

Altogether those data demonstrates that, by metabolizing DG, DGKs are decreasing TCR signaling and contributing to resistance to apoptosis in XLP-1 patients. Indeed DGKs knockdown restores *in vitro* TCR induced apoptosis in SAP deficient lymphocytes comparable to normal levels.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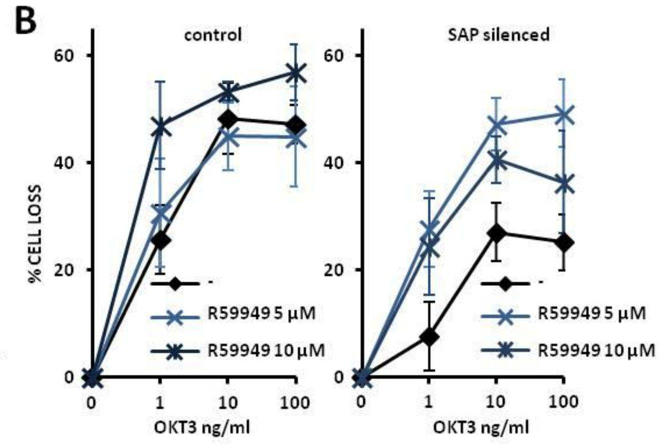
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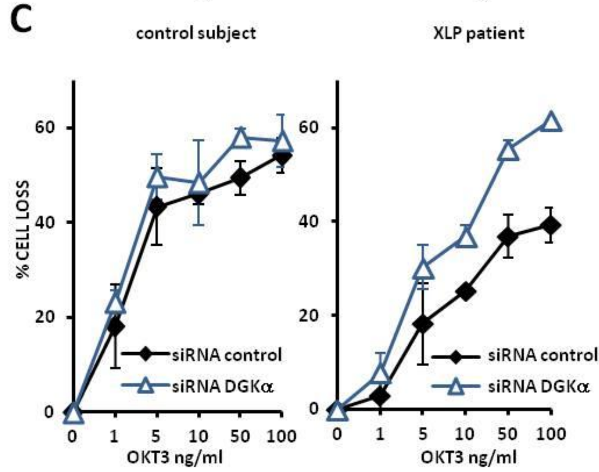
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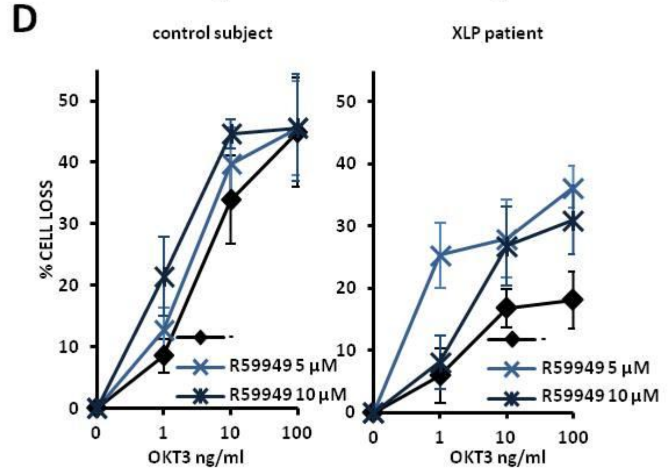
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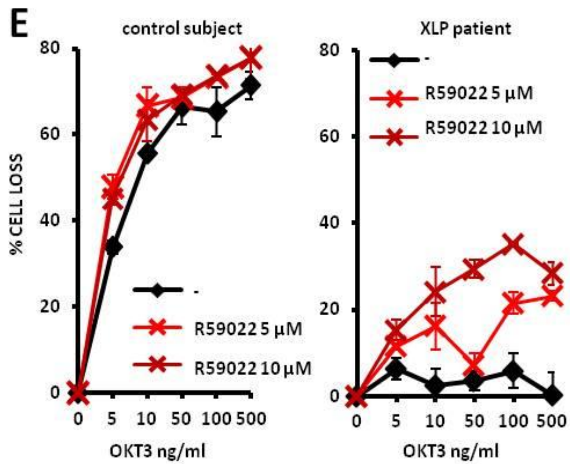
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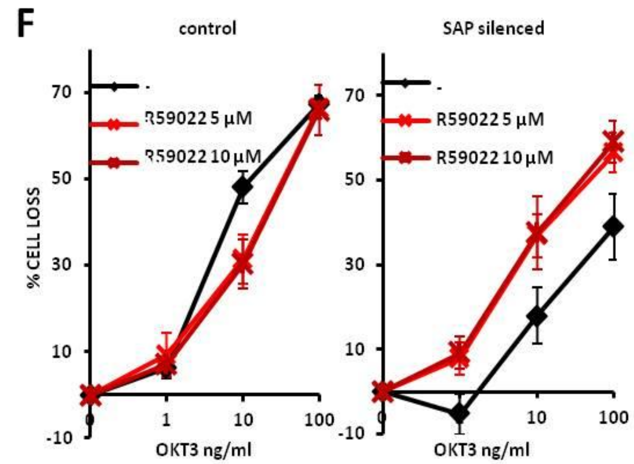
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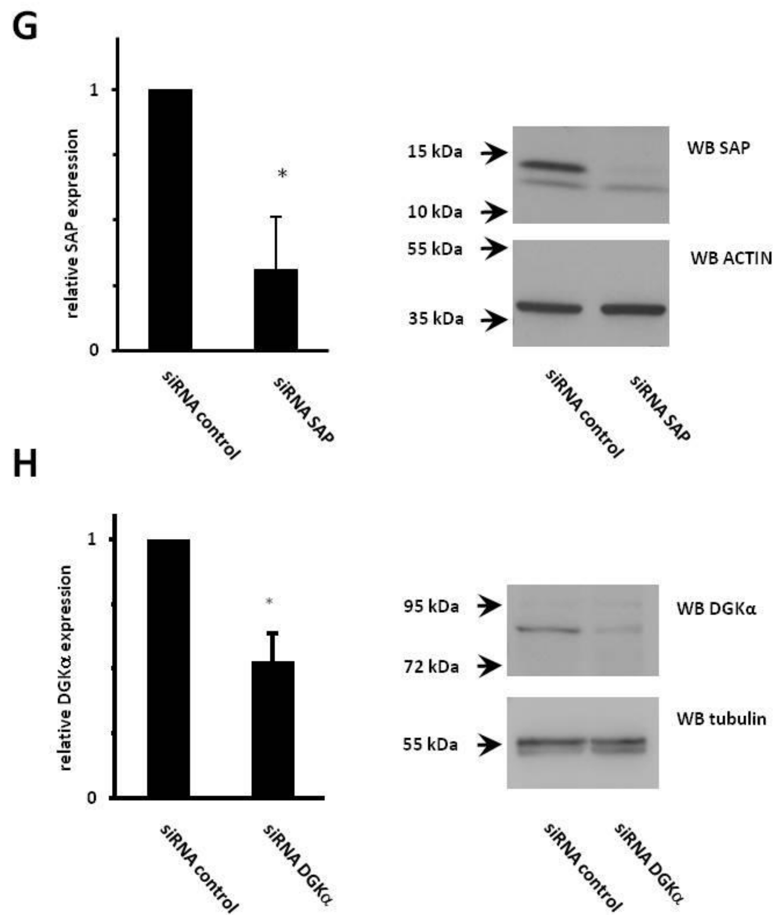

Fig.1 DGKα silencing or inhibition restores RICD in T cell both from XLP patients and in SAP deficient cells

A) Lymphocytes from normal subjects were transfected with the indicated siRNA and after 4 days restimulated with increasing doses of CD3 agonist OKT3. After 24 hours the % of cell loss was evaluated by PI staining. Data are the mean ± SEM of three independent experiments performed in triplicate.
B) Lymphocytes from normal subjects were transfected with the indicated siRNA and after 4 days restimulated with increasing doses of CD3 agonist OKT3 in presence or absence of DGK inhibitor R59949 (5 – 10 μM). After 24 hours the % of cell loss was evaluated by PI staining. Data are the mean ± SEM of five independent experiments performed in triplicate.
C) Lymphocytes from normal subject or XLP patient were transfected with the indicated siRNA and after 4 days restimulated with increasing doses of CD3 agonist OKT3. After 24 hours the % of cell loss was evaluated by PI staining. Data are the mean of two independent experiments performed in triplicate.
D) Lymphocytes from normal subject or XLP patient were restimulated with increasing doses of CD3 agonist OKT3 in presence or absence of DGK inhibitor R59949 (5 – 10 μM). After 24 hours the % of cell loss was evaluated by PI staining. Data are the mean ± SEM of three independent experiments performed in triplicate.
E) Lymphocytes from a normal subject or XLP patient were transfected with the indicated siRNA and after 4 days restimulated with increasing doses of CD3 agonist OKT3 in presence or absence of the DGK inhibitor R59022 (5 – 10 μM). After 24 hours the % of cell loss was

evaluated by PI staining. Data are the mean of three independent experiments performed in triplicate.

F) Lymphocytes were transfected with the indicated siRNA and after 4 days restimulated with increasing doses of CD3 agonist OKT3 in presence or absence of the DGK inhibitor R59022 (5 – 10 μM). After 24 hours the % of cell loss was evaluated by PI staining. Data are the mean ± SEM of five independent experiments performed in triplicate.

G) Lymphocytes were transfected with the indicated siRNA and after 4 days SAP expression was check by quantitative RT-PCR (left panel) or by western blot using actin as reference (right panel).

H) Lymphocytes were transfected with the indicated siRNA and after 4 days DGKα expression was check by quantitative RT-PCR (left panel) or by western blot using tubulin as reference (right panel).

2) Inhibition of DGKα restores normal DG dependent signaling in SAP deficient cells

Indeed SAP absence modify TCR signaling and decreases DG dependent signaling such as PKCθ[42] and the RasGRP1/Ras/MAPK pathway[11,40], whereas DGKα silencing or inhibition is known to potentiate DG signaling[43-45]. We thus we verify if DGKα knockdown could at least partially restore DG mediated signaling in SAP deficient primary lymphocytes. Several authors have reported that SAP absence results, *in vitro,* in an impairment of the induction of several TCR target genes such as IL-2 [40,46], IL-4[15], FASLG and BCL2L11[25] or p73[27] depending on the experimental system used.

We first focused on IL-2 and its receptor CD25 as their induction marks functional T cell activation but they are also required for activation induced apoptosis[47,48]. In line with a role of DGKα as a negative regulator of TCR signaling its silencing greatly potentiates IL-2 and INFγ transcription upon TCR triggering in control cells as reported by others [30]. Interestingly DGKα silencing compensates for SAP absence, bringing IL-2 and INFγ transcription back to normal levels in SAP deficient cells (Fig. 2A and 2B). Similarly SAP silencing decreases TCR induced IL-2 receptor expression, which is fully rescued upon DGKα silencing (Fig. 2C and 2D). This indicates that DGKα silencing can promote a functional signaling in SAP deficient cells and restores the IL-2/IL-2 receptor autocrine loop which sustains TCR proliferation but also contributes to RICD.

Surprisingly DGKα knockdown selectively rescues the TCR induced expression of just some SAP dependent genes. We finally focus on the orphan nuclear receptors Nur77 (NR4A1) and Nor-1 (NR4A3) which translocate to mitochondria mediating TCR induced cell death[49] and they are controlled by DGK activity[50]. We observed that SAP silencing, in lymphocytes from healthy donors, reduces TCR induction of both Nur77 and Nor-1 and a similar reduction is observed in lymphocytes of XLP patients (Fig. 2E and 2F), suggesting that their absence contributes to RICD resistance in XLP-1. Those data indicates that DGKα knockdown restores the expression of multiple TCR target genes involved in apoptosis and may at least partially compensate the signaling defects of SAP deficient cells.

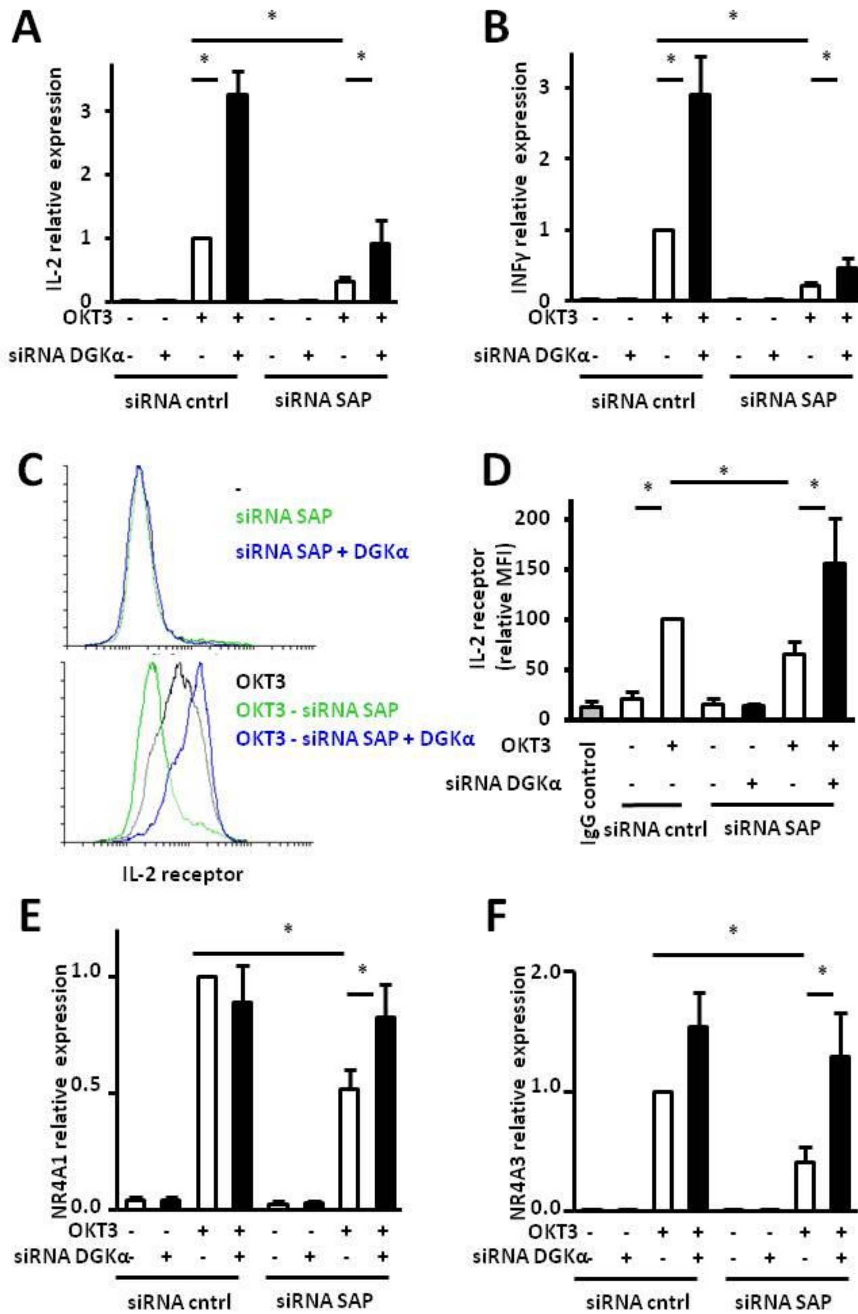

Fig.2 Silencing of DGKα restores DG dependent pro apoptotic signaling in SAP deficient cells

A) Lymphocytes were transfected with the indicated siRNA and after 4 days restimulated with CD3 agonist OKT3 (10 µg/ml 4 hours). IL-2 mRNA were quantified by quantitative RT-PCR using GUSB as reference gene. Graph shows the mean ± SEM of six independent experiments. * paired T test < 0.05.

B) Lymphocytes were transfected with the indicated siRNA and after 4 days restimulated with CD3 agonist OKT3 (10 µg/ml 4 hours). INFγ mRNA were quantified by quantitative RT-PCR using GUSB as reference gene. Graph shows the mean ± SEM of six independent experiments. * paired T test < 0.05.

C) Lymphocytes were transfected with the indicated siRNA and after 4 days were restimulated with CD3 agonist OKT3 (100 ng/ml 24 hours). IL-2 receptor α chain was quantified by cytofluorimetry. Histograms from a representative experiment is shown.
D) Graph presenting the mean ± SEM of IL-2 receptor α chain expression in five independent experiments. * paired T test < 0.05.
E) Lymphocytes were transfected with the indicated siRNA and after 4 days restimulated with CD3 agonist OKT3 (10 µg/ml 4 hours). NR4A1 mRNA were quantified by quantitative RT-PCR using GUSB as reference gene. Graph shows the mean ± SEM of six independent experiments. * paired T test < 0.05.
F) Lymphocytes were transfected with the indicated siRNA and after 4 days restimulated with CD3 agonist OKT3 (10 µg/ml 4 hours). NR4A3 mRNA were quantified by quantitative RT-PCR using GUSB as reference gene. Graph shows the mean ± SEM of six independent experiments. * paired T test < 0.05.

3) DGKα knockdown restores RICD by activating DG signaling through PKCθ and the RasGRP1 cascade.

Our data suggest that the defective RICD of SAP deficient cells is due to DG depletion by active DGKα. To demonstrate such hypothesis we treated PBLs before restimulation with the short chain 1,2-dioctanoyl-sn-glycerol (C8-DG) which is rapidly incorporated in cell membrane triggering DG signaling[52]. C8-DG treatment does not affect cell death in control cells, indicating that DG abundance is not limiting RICD in those cells. Conversely C8-DG significantly enhanced TCR induced cell death in SAP deficient cells, suggesting reduced levels of DG in this context (Fig. 3A).

To verify the involvement of PKCθ in RICD we silenced it alone or in combination with SAP and DGKα. We observed that silencing PKCθ decreases RICD in control cells confirming the reported relevance of this protein for lymphocyte activation induced cell death[53]. Conversely in SAP silenced cells, PKCθ silencing has no effects on the residual RICD, confirming that in those cells this kinase is inactive. Finally, in SAP and DGKα silenced cells RICD is rescued but the rescue is abrogated by PKCθ specific siRNA indicating that this kinase is critical for RICD restoring by DGKα inhibition (Fig. 3B).

To verify the involvement of RasGRP1 in RICD we silenced it alone or in combination with SAP and DGKα. We observed that silencing RasGRP1 decreases RICD in control cells confirming the reported relevance of this protein for lymphocyte activation induced cell death[54]. Conversely in SAP silenced cells RasGRP1 silencing has no effects on the residual RICD, confirming that in those cells this kinase is inactive. Finally, in SAP and DGKα silenced cells RICD is rescued but the rescue is abrogated by RasGRP1 specific siRNA indicating that this GEF is critical for RICD restoring by DGKα inhibition (Fig. 3C). Altogether those data indicates that, in SAP deficient cells, DGKα silencing restores an efficient DG signaling.

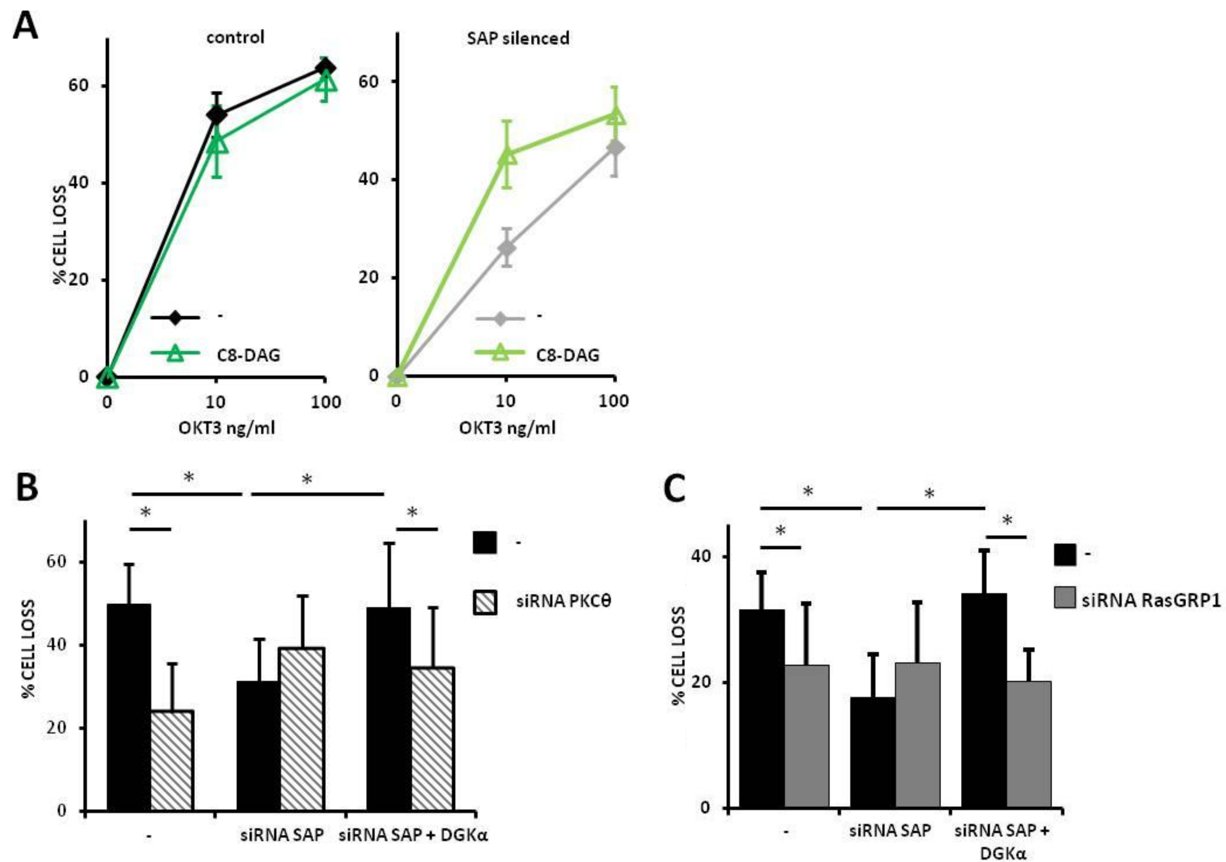

Fig.3 DGKα silencing rescues RICD in SAP deficient cells by activating PKCθ and the RasGRP1-MAPK pathway

A) Lymphocytes were transfected with the indicated siRNA and after 4 days restimulated with increasing doses of CD3 agonist OKT3 in presence or absence of C8-DAG (50 μM). After 24 hours the % of cell loss was evaluated by PI staining. Data are the mean ± SEM of five independent experiments performed in triplicate.

B) Lymphocytes were transfected with the indicated siRNA and after 4 days were restimulated with CD3 agonist OKT3 (10 ng/ml). After 24 hours the % of cell loss was evaluated by PI staining. Data are the mean ± SEM of six independent experiments performed in triplicate. * paired T test < 0.05.

C) Lymphocytes were transfected with the indicated siRNA and after 4 days were restimulated with CD3 agonist OKT3 (10 ng/ml). After 24 hours the % of cell loss was evaluated by PI staining. Data are the mean ± SEM of six independent experiments performed in triplicate. * paired T test < 0.05.

5. Discussion

Based on our previous observations that SAP mediated inhibition of DGKα is required for efficient TCR signaling [40] and that in SAP absence TCR signaling is reduced and does not reach the threshold required for RICD onset [25], we speculated that the lack of DGKα inhibition by SAP, reducing TCR signaling, contributes to resistance to TCR promoted cell death. In this scenario DGKα knockdown should restore TCR signaling and TCR induced apoptosis and possibly be beneficial in XLP treatment.

Indeed we found that DGKα silencing or inhibition does not affect TCR triggered cell death in control cells but bring it back to normal levels in SAP silenced T cells or lymphocytes from XLP patients (Fig. 1A to 1F).

6. References

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7. Attended seminars

SPEAKER	TITLE	DATE
Prof. SALVATORE OLIVIERO Department of Life Sciences and System Biology, Università di Torino & HuGeF	" *Epigenetic modifications that control stem cell differentiation*"	19/02/2014
Prof. EMILIO HIRSCH Dipartimento di Biologia Molecolare e Cellulare e Genetica Molecolare, Università di Torino	" *Role of Phosphoinositides-3-kinase C2-alpha, a Class II PI 3-kinase, in development and cancer*"	19/03/2014
Miltenyi Biotec Mini MACS Simposio Dipartimento di Scienze della Salute, Università del Piemonte Orientale "A.Avogadro"	***"Il ruolo emergente delle vescicole extracellulari in fisiopatologia: da mediatori cellulari e biomarker"***	12/05/2014
Prof. Antonia Follenzi Dipartimento di Scienze della Salute, Università del Piemonte Orientale "A.Avogadro"	"***Terapia Genica"***	19/06/2014
Prof. GIANNI DEL SAL Department of Life Sciences, Università di Trieste LNCIB	"***Disarming mutant P53 in Cancer***"	26/06/2014
Dott. Diego Cotella Dipartimento di Scienze della Salute, Università del Piemonte Orientale "A.Avogadro"	***"The C-value paradox, junk DNA and ENCODE"***	30/06/2014
Prof. Antonia Follenzi Dipartimento di Scienze della Salute, Università del Piemonte Orientale "A.Avogadro"	"***Applicazioni Terapia Genica"***	14/07/2014
Dr. Maria Giuseppina Miano Institute of Genetics and Biophysics ABT, CNR- Napoli	***"A functional link between ARX and KDM5C genes linked to neurophenotypes defines a crucial disease path"***	21/07/2014
Prof. Steven R. Ellis Department of Biochemistry and Molecular Biology, University of Louisville (Kentucky, USA)	***"The Borghese Sessions"***	8-22/09/14

8. Partecipations to conferences

- 3rd- 4th November 2014, A symposium for researcher and clinicians on XLP and WAS, London, England.
Inhibition of Diacylglycerol kinase alpha rescues TCR-induced diacylglycerol signaling and restimulation induced cell death in XLP lymphocytes

- 23rd -24th October 2014, SIICA - SSAI Joint Workshop
"Imaging the Immune system", San Raffaele, Milan, Italy.

"Inhibition of Diacylglycerol kinase alpha rescues restimulation induced cell death in XLP T lymphocytes and prevents aberrant CD8+ expansion in LCMV-infected SAP -/- mice"
V. Malacarne*, **E. Ruffo*,** L. Patrussi, K.E. Nichols, I. Rubio, A.L. Snow, G. Baldanzi and A. Graziani.

-9th-13th June 2014, SIB, Società Italiana di Biochimica e Biologia Molecolare
"26a RIUNIONE NAZIONALE *"A. Castellani"* DEI DOTTORANDI DI RICERCA IN DISCIPLINE BIOCHIMICHE", Brallo di Pregola, Italy.
"SAP-MEDIATED INHIBITION OF DIACYLGLYCEROL KINASE ALPHA REGULATES TCR-INDUCED DIACYLGLYCEROL SIGNALING AND RESTIMULATION INDUCED APOPTOSIS IN XLP PATIENTS"
E.Ruffo, G.Baldanzi, V.Malacarne, K.E. Nichols, L.Patrussi, C.T Baldari, I.Rubio, C. Wuelfing, A.L. Snow and A.Graziani.

- 17th-21st May 2014, EMBO conference
"Lymphocyte Signalling", Bertinoro, Italy.
"SAP-MEDIATED INHIBITION OF DIACYLGLYCEROL KINASE ALPHA REGULATES TCR-INDUCED DIACYLGLYCEROL SIGNALING AND RESTIMULATION INDUCED APOPTOSIS IN XLP PATIENTS"
E.Ruffo, G.Baldanzi, V.Malacarne, K.E. Nichols, L.Patrussi, C.T Baldari, I.Rubio, A.L. Snow and A.Graziani.

- 5th-8th May 2014, 50th FEBS, Biochemistry society- Advancing Molecular Bioscience
"Membrane, Morphology and Function", Hotel del Camerlengo, Fara San Martino, Abruzzo, Italy.
1) "SAP-MEDIATED INHIBITION OF DIACYLGLYCEROL KINASE ALPHA REGULATES TCR-INDUCED DIACYLGLYCEROL SIGNALING AND RESTIMULATION INDUCED APOPTOSIS IN XLP PATIENTS"
E.Ruffo, G.Baldanzi, V.Malacarne, K.E. Nichols, L.Patrussi, C.T Baldari, I.Rubio, A.L. Snow and A.Graziani.
2) "The Diacylglycerol Kinase α/ Atypical PKC/ β1 Integrin Pathway in SDF-1α Mammary Carcinoma Invasiveness"
G.Baldanzi, E.Rainero, C.Cianflone, P.E. Porporato, F.Chianale, **E.Ruffo**, V.Malacarne, J.C. Norman, F.Sinigaglia and A.Graziani.

- 29th March 2014, ABCD (Associazione di Biologia Cellulare e del Differenziamento)
"Forum stem cell therapy: hype or hope?", Lugano, Switzerland.

9. Publications

- The diacylglycerol kinase α/atypical PKC/β1 integrin pathway in SDF-1α mammary carcinoma invasiveness.

Rainero E, Cianflone C, Porporato PE, Chianale F, Malacarne V, Bettio V, **Ruffo E**, Ferrara M, Benecchia F, Capello D, Paster W, Locatelli I, Bertoni A, Filigheddu N, Sinigaglia F, Norman JC, Baldanzi G, Graziani A.
PLoS One. 2014 Jun 2;9(6):e97144. doi: 10.1371/journal.pone.0097144. eCollection 2014.